

Curcumin Improves the Behavior and Memory in Mice by Modulating the Core Circadian Genes and Their Associated Micro-RNAs

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Abstract

Objective: To investigate the neuroprotective effects of curcumin in albino mice focusing on memory and behavior. **Materials and Methods:** Four groups of 7 mice each, different concentrations of curcumin were administered for 3 weeks in 3 groups and the remaining group was kept as control. The effect of curcumin on behavior and memory was studied using various tests. The expression of core circadian rhythm genes and micro RNAs were also studied. **Results:** Our results revealed that curcumin improves the mice's behavior by regulating oxidative stress, hippocampal neurogenesis, and modulation of core circadian genes and their associated micro-RNA. **Conclusion:** This study shows that curcumin has a potential neuroprotective effect.

Keywords: Brain-derived neurotrophic factor, BMAL1, circadian rhythm, CLOCK, curcumin, DCX, MiRNA, SIRT1, synapsin

INTRODUCTION

Our daily circadian timing system plays an important role in brain development. Suprachiasmatic nucleus (SCN) of the hypothalamus is the master circadian clock and primary pacemaker in mammals. SCN contains the molecular machinery necessary for self-sustaining circadian oscillations.^[1] The SCN transforms the external environmental light/dark information into internal neural and endocrine signals that regulate the peripheral clock.^[2] The circadian clock generates a 24-h rhythm through which we adapt our physiology and behavior to daily changes in the environment. Many biological processes such as hormone secretion and sleep–wake cycles are controlled by the circadian clock.^[3] Therefore, an innate malfunctioning of the circadian clock or a shift between internal circadian rhythm and the external environment can cause various pathologies. Sleep disorders, altered metabolism, obesity, diabetes, mood disorders, cancer, and cardiovascular diseases are all linked to an abnormal circadian rhythm.^[4]

The core circadian oscillator comprises several circadian genes including Circadian Locomotor Output Cycles Kaput (*CLOCK*), Brain and Muscle ARNT (Aryl hydrocarbon Receptor Nuclear

Translocator) like protein 1 (*BMAL1*), Cryptochrome (*Cry*), Period (*Per*), retinoic acid orphan related (*ROR*), reverse erythroblastomas α -virus (*REV-ERB α*), and casein kinase 1 epsilon (*ck1 ϵ*).^[5] These genes regulate the daily circadian rhythm through positive/negative transcriptional/translational feedback loop (TTFL) of their downstream protein production.^[5] Peripherally, each cell of our body contains local clock genes, and the central SCN controls these peripheral oscillators which locally control the overt rhythmic expression of protein and hormones.^[5] In the positive arm of one of the TTFLs, *CLOCK* and *BMAL1* form heterodimers and positively regulate the expression of clock-controlled genes including *Per* and *Cry*.^[6] In the negative arm of the loop, the *PER:CRY* complex along with *ck1 ϵ* translocates into the nucleus, interacts with

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CLOCK: BMAL1, and inhibits the transcriptional activity of CLOCK: BMAL1, thereby repressing the transcription of *Per* and *Cry* and other clock-controlled genes. After the *PER* and *CRY* proteins are degraded, the transcription repression gets relieved and a new cycle starts.^[5]

The rhythmic expression of these core circadian genes regulates the various kinds of emotions in the neurodegenerative disorders such as Parkinson's disease,^[7] schizophrenia and depression,^[8] metabolic dysfunctions, mood disorders, and cognitive impairment in nocturnal and diurnal species.^[9] Apart from that, several other researchers have also reported that the prevalence of major stress and depressive-like disorders is higher in night shift workers.^[10] However, the 24 × 7 occupational work system became a routine feature of modern society and people tend to continuously work and socialize round-the-clock, which has tremendous health hazards and it is crucial time to search for a natural remedy to tackle the challenges of mental dementia in today's competitive world.

Many natural products have proven health benefits and neuroprotective effects.^[11] Curcumin, which is a natural polyphenol of *Curcuma longa*, also holds a great potential because of its immense medicinal properties. Many researchers have reported its neuroprotective role both in the *in vitro* and *in vivo* models of neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, multiple sclerosis, Huntington's disease, schizophrenia, and depression.^[12] Most of these neurodegenerative diseases are caused by either oxidation or inflammation through excessive production of reactive oxygen species or free radicals. Since curcumin can cross the blood-brain barrier, it is a very effective therapeutic herbal medicine against neurodegenerative diseases. Besides having poor bioavailability,^[13] it can regulate several key modulators of dentate gyrus neurogenesis including Brain-derived neurotrophic factor (BDNF) and cAMP response-element binding protein (CREB) proteins. Several researchers have reported that oral administration of curcumin improves the spatial learning and retention memory in rats and mice.^[14] Curcumin also plays a protective role against chronic unpredictable stress-induced cognitive impairment and oxidative damage in mice neuronal cells.^[15]

Researchers have shown a wide range of neuroprotective effects of curcumin, but the specific role of curcumin in the improvement of cognitive and noncognitive behavior of mice through modulation of circadian clock genes is yet unexplored. The present study focuses on the health promotive effects of curcumin in Swiss Albino mice encompassing behavioral studies (open-field test, novel object recognition (NOR) test, and Morris water maze (MWM) test) and biochemical analyses. We also investigated the expression levels of hippocampal proteins, which are involved in neurogenesis (BDNF, Synapsin II, and DCX). Further, we monitored the expression levels of core circadian genes (*SIRT1*, *BMAL1*, and *CLOCK*) and their associated miRNAs (miRNA21a-5p and miRNA34a-5p). We identified the effect of curcumin that decreases the interaction

between *CLOCK* and *BMAL1* interfering with the expression of the *CLOCK* gene by *in vivo* studies.

MATERIALS AND METHODS

Animals

For the present study, twenty-eight Swiss Albino mice (4 weeks old; weighing 25–30 g) were obtained from the animal house of Amity University, Noida. The animals were housed individually in propylene cages (dimensions: 33 cm × 19 cm × 14 cm) at the ambient temperature of 22°C ± 2.4°C. For sustenance, they were provided Harlan Tekla 8640 food (Madison, WI) and filtered tap water *ad libitum*. A standard light-dark cycle (LD; 12:12 light [~150 lux]/dark [0 lux]) was maintained using a lux meter during the entire 3 weeks of experimentation. All the experimental procedures conducted as part of this study were duly approved by the Institutional Animal Care and Ethical Committee of Amity University. The recommendations cited by the Committee for Control and Supervision of Experiments and Animals (CPCSEA), India, were used as guidelines for maintaining the mice during this period. After 1-week of acclimatization, the mice were randomly assigned to experimental groups and transferred to a cabinet.

Drugs and biochemical reagents

The curcumin extract was purchased from Sisco Research Laboratory (SRL) Pvt. Ltd., India. Phosphate buffer saline (PBS), formaldehyde, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), trichloroacetic acid, thiobarbituric acid (TBA), butanol, nitroblue tetrazolium (NBT), disodium hydrogen phosphate, sodium citrate, and hydrogen peroxide (H₂O₂) were obtained from Thermo Fisher Scientific, MA, USA. The mouse brain-derived neurotrophic factor (BDNF) ELISA kit and Mouse CREB ELISA kit were purchased from Ray Biotech, Georgia, USA, while the Mouse Synapsin II ELISA kit and mouse DCX ELISA kit were purchased from Gentaur, Belgium. Polytron tissue homogenizer (Thomas Scientific, India), ELISA reader (Trans ASIA Pvt., Ltd., India), and ultraviolet (UV) spectrometer (Perkin Elmer, Waltham, MA, USA) were used. Primers were synthesized from Geno Biosciences, India.

Experimental design and drug treatment

Curcumin extract 90% was dissolved in 1% carboxymethyl cellulose (CMC) for experimentation. As described earlier, the animals were divided into four groups (*n* = 7 per group). These comprised as follows:

1. Control group (1% CMC) (*n* = 7)
2. Cur50 (curcumin 50 mg/kg) (*n* = 7)
3. Cur100 (curcumin 100 mg/kg) (*n* = 7)
4. Cur150 (curcumin 150 mg/kg) (*n* = 7).

Carboxymethyl cellulose (CMC) and curcumin were administered orally to the 4-week-old mice once a day for 3 weeks. The details of the experimental design are depicted in Table 1.

Behavior studies

Open-field test

The open-field test (OFT) apparatus contained a square arena (40 cm × 40 cm) that was divided into 4 center squares and 16 outer squares. It was placed in a medially lit room (~20 lux) and a video recording device was kept above the square arena for documentation and observation purposes. The animals were placed in the center of the OFT apparatus and allowed free access to explore the area for 5 min in each trial. The subsequent analysis was undertaken by following defined parameters which accounted for the total number of crossings from one square to another, the number of entries in the center square, time spent in the center square, rearing frequency (number of times the animal stands on their hind paws). The line crossing was considered only when an animal entered another square with all of its four paws. Between each test, the OFT apparatus was cleaned with a 10% ethanol-water solution to neutralize any odors left by the other mouse.^[16]

The novel object recognition test

The NOR apparatus comprised a square-shaped open field box made of black Plexiglass with dimensions of 40 cm × 40 cm. Two familiar objects were placed on the opposite side of the starting point. The mice were allowed free access to explore these two identical objects (cylinders) in the apparatus for 5 min in each trial (learning period). This was facilitated two times a day (morning and evening) for 2 successive days. On the 3rd day, one of the familiar objects was replaced by a novel object (cuboid), and the mice were allowed to explore both of these two objects (novel and familiar) for 2 min in each test. The time that the mice spent on exploring the familiar object (T1) and novel object (T2) was noted for the analysis of recognition memory index.^[17] The recognition memory was measured using the object recognition index, wherein the ratio of time spent to explore the novel object and time spent to explore the familiar object (T2/T1) was calculated.

Morris water maze test

The MWM test^[18] apparatus comprised a large circular pool (130 cm in diameter and 60 cm height) filled with warm and cloudy water to a depth of 30 cm. In the first quadrant, a target platform was kept hidden at a depth of 2 cm below the surface of the water. Each mouse was randomly released

in the pool facing a wall at one of the four points (east, west, south, or north) and provided up to 90 s (s) to locate the invisible platform. Once the mouse successfully located the platform (or was guided to the platform after the period of 90 s), the animal was given a period of 10 s to remain on the platform. The video recording device documented the total time taken to find the hidden platform and the time spent in the correct platform quadrant. For 5 days, the acquisition of memory retention and cognitive functions was measured in a set of four trials per day.

Tissue sample collection

After the behavior test, sodium thiopental (50 mg/kg) was used to anesthetize the mice. They were sacrificed and the hippocampus was dissected out on ice-cold surgical plates. PBS (pH 7.4) was used to homogenize the hippocampus tissue samples from different groups and subsequently centrifuged at 10,000 rpm for 15 min. The supernatants were collected for further biochemical and protein analyses.

Estimation of oxidative stress

Lipid peroxidation

The quantitative study of the lipid peroxidation in the hippocampal region of different mice groups was undertaken using the protocol laid down by Wills.^[19] Each brain sample was mixed with 1 ml of 10% trichloroacetic acid and 1 ml of 0.67% thiobarbituric acid. This was heated in a boiling water bath for 15 min and butanol (2:1 v/v) was added. Using a UV spectrophotometer, the level of malondialdehyde (MDA) was analyzed by reaction with TBA at 532 nm. The total amount of MDA was derived using the molar extinction coefficient of MDA-TBA adduct at 532 nm is 155 (nM/cm).

Superoxide dismutase activity

The NBT method was used to analyze superoxide dismutase activity (SOD). This method is derived from the principle that NBT goes through photoreduction (which is a blue-colored formazan) when exposed to light. NBT competes with the SOD enzyme for superoxide anions. When SOD is present in the reaction mixture, there is reduced production of the colored complex by NBT as compared to control. The absorbance obtained from NBT reduction to blue formazan by superoxide was determined at 560 nm.^[20]

Catalase activity

The catalase (CAT) enzyme activity was quantitatively assessed using the protocol of Luis *et al.*^[21] Briefly, the brain homogenate (1 ml) was transferred into a test tube and 1.9 ml of PBS (50 mM, pH 7.4) was added to it. To initiate the reaction, 1 ml of 30 mM H₂O₂ was added to the mixture. A mixture of 2.9 ml of PBS and 1 ml of H₂O₂ without a brain homogenate was taken as blank. H₂O₂ decomposition resulted in reduced absorbance, which was recorded at 240 nm. The unit of CAT activity was quantitatively expressed as the amount of enzyme that decomposes 1 μM of H₂O₂ per minute at 25°C using the molar coefficient of 43.6 M/cm. This activity was expressed as unit/mg proteins.

Table 1: Detail of experimental design

Groups	Number of mice (n)	Experiment conditions	Treatment	Duration (weeks)
Control	7	12:12 light (~150 lux)/dark (~0 lux)	1% CMC	3
Cur50	7	12:12 light (~150 lux)/dark (~0 lux)	50 mg/kg curcumin	3
Cur100	7	12:12 light (~150 lux)/dark (~0 lux)	100 mg/kg curcumin	3
Cur150	7	12:12 light (~150 lux)/dark (~0 lux)	150 mg/kg curcumin	3

CMC=Carboxymethyl cellulose

Hippocampus protein estimation

The hippocampal tissue dissected out from the experimental animals was used to study three proteins, namely BDNF, Synapsin II, and DCX. 1 mg tissue was mixed with 300 μ l of lysis buffer and subsequently homogenized for a period of 30 s and then centrifuged at 10,000 rpm for 15 min at 4°C. All the samples were assayed in triplicate and an ELISA plate reader (Trans Asia Pvt., Ltd., India) was used to record the absorbance (450 nm). For each protein sample, the concentration was computed by plotting the absorbance values on a bovine serum albumin standard curve as generated through the assay.

RNA extraction and cDNA synthesis

Following the established protocol laid down by Sarwat and Naqvi^[22] the total RNA was isolated from the frozen mice's brain tissues. The TRIzol method was used wherein RNA was dissolved in 30 μ l nuclease-free water and quantified by NanoDrop (NanoDrop Technologies, Wilmington, DE). The concentration of RNA was calculated at 260 nm and purity assessment was recorded at 260 nm/280 nm of the absorbance values. For qualitative analysis, RNA was analyzed by 1% agarose gel electrophoresis. The cDNA synthesis was processed by the high-capacity cDNA kit (Thermo Fisher). In brief, the RNA was reverse transcribed following the manufacturer's instructions, wherein 1000 ng of total RNA and $\times 10$ RT primer was used. The synthesized cDNA was stored in -20°C freezers for further testing.

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (PCR) was performed separately for mRNA of *CREB*, *BDNF*, *Synapsin II*, and *DCX* along the circadian genes *CLOCK*, *BMAL1*, and *SIRT1* and the respective vehicle control groups. In each reaction, 1 μ l (~50 ng) cDNA was employed with gene-specific forward and reverse primers [Table 2] and SYBER Green master mix (Applied Biosystems, Foster City, CA, USA). A StepOne™ real-time PCR System (Applied Biosystems, Foster City, CA, USA) was used for the assessment of the samples. Along with the individual gene of interest, β -actin was run as a reference control to normalize gene expression. The reaction procedure entailed a time duration of 10 min at 95°C, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. All of the above-mentioned experiments were run in triplicate, along with respective negative and positive controls. In comparison to the normal controls, the relative gene expression fold change for each sample was calculated based on the threshold cycle (CT) value using the following formula: relative quantification (RQ) = $2^{-\Delta\Delta CT}$.

miRNA profiling in brain tissue using SYBER Green

To analyze the expression fold change of miR-21a-5p and miR-34a-5p, RT-PCR was performed individually along with the respective controls. For this purpose, miRNA-specific forward and reverse primers [Table 3] and SYBER Green master mix (Applied Biosystem, Foster City, CA, USA) were used. Small nuclear RNA U6 was kept as a reference control and the default threshold settings were used to ascertain the threshold

cycle. In each miRNA analysis, the concentration of cDNA used was uniform to maintain efficiency. The reaction profile was 10 min at 95°C, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min. Each of these experiments was run in triplicate mode along with corresponding negative and positive controls. In the case of each sample, the miRNA expression fold changes in comparison to normal control were calculated. This was done based on CT value using the following formula: $RQ = 2^{-\Delta\Delta CT}$.

statistical analysis

The values were represented as mean $\pm$ standard deviation ($n = 7$). One-way ANOVA with a *post hoc* Duncan's test was employed to compare the results between LD versus LD + Cur50, LD + Cur100, and LD + Cur150. All the data were analyzed in GraphPad Prism-8. The values of $P < 0.05$ represented a statistically significant difference between the groups.

RESULTS

Behavior studies

Curcumin improves the noncognitive behavior of mice

The mean number of lines crossed, center square entry, center square duration, and rearing are depicted in Table 4. The open-field result reflected that the noncognitive behavior like locomotor activity and depressive-like behavior was significantly improved in the curcumin-treated mice groups as compared to the vehicle control group.

The mean number of lines crossed was significantly higher in the curcumin-treated group as compared to the control group ($F_{3,12} = 22.46$, $P < 0.001$). The increase in line crosses in curcumin-treated mice groups showed a dose-dependent pattern. This exhibits that curcumin has the potential to improve the locomotor activity of mice. Moreover, the center square entry ($F_{3,12} = 5.42$, $P < 0.01$) and durations ($F_{3,12} = 15.70$, $P < 0.001$) were also higher in curcumin-treated groups of mice as compared to control group and this shows that curcumin has the therapeutic effect of improving the stress and depressive-like behavior of mice. However, there is no significant difference in the case of rearing behavior between control and curcumin-treated groups, but curcumin-treated mice showed a slightly increased number of rearing in a dose-dependent manner, hence exhibiting improvement in the anxiety-like behavior of mice after treatment with curcumin.

Curcumin elevates the spatial learning and retention memory of mice

In the present study, the result of the MWM test shows that curcumin-treatment improves spatial learning and retention memory as compared to the control group. The time (mean) spent in finding the hidden platform (of MWM tank) was reduced when the mice were treated with curcumin as compared to the control untreated mice ($F_{3,12} = 48.53$, $P < 0.001$), thus exhibiting the potentially positive effect of curcumin on the spatial learning of mice. This positive effect is dose-dependent as shown in Table 4. Moreover, the total time spent in the platform quadrant,

Table 2: Sequences of quantitative/real-time polymerase chain reaction primer pairs

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
BDNF	GAAGAGCTGCTGGATGAGGAC	TTCAGTTGGCCTTTGGATACC
Synapsin II	TCAGCAAGATGAACCAGC	GACTTGTGAGCTGTGGG
DCX	TGCTCAACCCAGAGAGAACA	CTGCTTCCATCAAGGGTGT
CREB	CCAAACTAGCAGTGGGCAGT	GAATGGTAGTACCCGGCTGA
CLOCK	CCTATCCTACCTTCGCCACACA	TCCCGTGGAGCAACCTAGAT
BMAL1	CCAAGAAAGTATGGACACAGACAAA	GCATTCTTGATCCTTCCTTGGT
SIRT1	AGTTCAGCCGTCTCTGTGT	CTCCACGAACAGCTTCACAA
β -actin	TGGTGGGTATGGGTCAGAAGGACTC	CATGGCTGGGGTGTGAAGGTCTCA

Table 3: Sequences of quantitative/real-time primers for microRNA

microRNA Name	Forward primer (5'-3')	Reverse primer (5'-3')
U6	GCTTCGGCAGCACATACTAAAT	CGCTTCACGAATTTGCGTGCAT
miRNA-34a-5p	GGTGGCAGTGCTTAGCT	CAGTGCCTGTCGTGGAGT
miRNA-21a-5p	GGGGGGTAGCTTATCAGACTG	CAGTGCCTGTCGTGGAGT

Table 4: Effect of curcumin on behavioral and biochemical alterations in mice

Behavioral parameters								
Test	Parameters	Group				One-Way ANOVA		
		Control	Cur50	Cur100	Cur150	F	df	P
OFT	NLC (n)	152.75±5.50	158.75±3.50 ^a	164.75±4.57 ^b	172.00±2.94 ^c	22.46	12	P<0.001
	CSE (n)	06.00±0.81	7.25±0.95	7.75±0.95 ^a	8.25±0.50 ^b	5.424	12	P<0.01
	CSD (s)	12.48±0.55	13.62±0.47 ^a	14.18±0.49 ^b	14.60±0.31 ^c	15.70	12	P<0.001
	RR (n)	45.25±1.70	45.75±0.50	46.50±1.29	47.25±0.95	2.130	12	P>0.05
MWM	TSFP (s)	42.25±0.95	40.00±1.41 ^a	37.00±0.81 ^b	34.00±0.81 ^c	48.53	12	P<0.001
	TSPQ (s)	49.50±1.29	50.75±1.70	55.25±0.96 ^a	58.00±0.82 ^b	40.49	12	P<0.001
NOR	T2/T1	1.24±0.01	1.43±0.03 ^a	1.52±0.01 ^b	1.59±0.01 ^c	253	12	P<0.001
Biochemical parameters								
	MDA (nM/mg)	2.10±0.01	1.71±0.07 ^a	1.45±0.06 ^b	1.30±0.05 ^c	157.8	12	P<0.001
	SOD (Unit/mg)	6.46±0.31	7.63±0.15 ^a	8.46±0.20 ^b	9.37±0.22 ^c	115.7	12	P<0.001
	CAT (Unit/mg)	3.37±0.08	3.84±0.20 ^a	4.46±0.16 ^b	5.17±0.13 ^c	179.6	12	P<0.001

Values are represented as mean±SD (n=7). One-Way ANOVA with a *post hoc* Duncan's test was employed to compare the results between control versus curcumin treatment group. Mean values bearing the dissimilar alphabets are statistically significantly different at group level (P<0.001). OFT=Open-field test; MWM=Morris water maze; NOR=Novel object recognition; NLC=Number of line cross; CSE=Center square entry; CSD=Center square duration; RR=Rearing; TSFP=Time spent to find platform; TSPQ=Time spent in platform quadrant; T1/T2=Time spent with novel object/Time spent with familiar object; MDA=Malondialdehyde; SOD=Superoxide dismutase; CAT=Catalase; n=Number; s=Second, SD=Standard deviation

a measure of retention memory, was also found to be higher in the curcumin-treated groups of mice in comparison to the control group [Table 4]. Statistically significant differences ($F_{3,12} = 40.49$, $P < 0.001$) of retention memory between the control group and curcumin-treated groups can be seen.

Curcumin enhanced the recognition memory of mice

The NOR test measured by the recognition index [ratio of time spent with novel objects (T2) and time spent with familiar objects (T1)]. In the present study, the NOR test revealed that curcumin has a positive effect on the recognition memory (T1/T2) of rodents in a dose-dependent manner [Table 4]. A statistically significant difference ($F_{3,12} = 253.00$, $P < 0.001$) of recognition memory between the control and curcumin-treated groups can be seen.

Biochemical

Curcumin reduces the lipid peroxidation and increases the endogenous antioxidant enzymes

In the present study, the mean level of MDA (a measure of lipid peroxidation) is reduced dose-dependently in curcumin-treated groups as compared to the control group [Table 4]. A statistically significant difference ($F_{3,12} = 157.80$, $P < 0.001$) in the MDA level can be seen between the control and curcumin-treated groups. This finding shows that curcumin might reduce oxidative stress by decreasing lipid peroxidation.

SOD and CAT are endogenous antioxidant enzymes that help in fighting with the reactive oxygen species of our body. In our study, we have observed that curcumin treatment has

effectively increased these enzyme levels in a dose-dependent manner [Table 4]. A statistically significant difference in the level of SOD ($F_{3,12} = 115.70$, $P < 0.001$) and CAT ($F_{3,12} = 179.6$, $P < 0.001$) between the control and curcumin-treated groups can be seen. Curcumin treatment increases the production of endogenous antioxidant enzyme SOD.

Together, these findings postulate that curcumin might be involved in protecting the neuronal cells from oxidative stress either by the production of SOD and CAT enzymes or reducing the MDA level.

Curcumin promotes neurogenesis through elevation of hippocampal proteins and genes

The extent of hippocampal neurogenesis is dependent on the level of hippocampal proteins including BDNF, Synapsin II, DCX, and CREB. Our results revealed that the treatment of different concentrations of curcumin increased the expression level of hippocampal BDNF ($F_{3,20} = 782.00$, $P < 0.001$), Synapsin II ($F_{3,20} = 209.90$, $P < 0.001$), and DCX ($F_{3,20} = 182.90$, $P < 0.001$) proteins in the curcumin-treated groups as compared to the control group [Figure 1a-c]. Moreover, the expression level of these genes (mRNA) was also significantly increased in the curcumin-treated groups [Figure 2a-c]. Further, the expression level of hippocampal CREB mRNA is also significantly increased in curcumin-treated groups ($P < 0.001$) [Figure 2d]. Therefore, our results indicate the important role of curcumin in the promotion of hippocampal neurogenesis (modulated by BDNF and CREB) and synaptogenesis (modulated by Synapsin II). Further, increased levels of DCX in curcumin-treated groups give us an indication of the increased production of hippocampal neural progenitor cells.

Curcumin modulates the expression of core circadian genes and their associated micro-RNA

The expression levels of *SIRT1* ($F_{3,8} = 609.10$, $P < 0.001$) and *BMAL1* ($F_{3,8} = 227.20$, $P < 0.001$) were significantly increased in the curcumin-treated groups of mice in a dose-dependent manner [Figure 3a and b]. However, the expression level of the *CLOCK* ($F_{3,8} = 220.70$, $P < 0.001$) gene was decreased in the curcumin-treated group in a

dose-dependent manner [Figure 3c]. This finding reveals that curcumin has a potential modulatory effect on the expression of core circadian genes. We have also monitored the changes in the expression level of two circadian gene associated miRNAs, viz., *CLOCK*-associated miRNA21a-5p and *SIRT1*-associated miRNA34a-5p [Figure 4a and b]. The result revealed that treatment of curcumin had increased the expression of miRNA21a-5p ($F_{3,8} = 486.6$, $P < 0.001$) and miRNA34a-5p ($F_{3,8} = 89.69$, $P < 0.001$) in a dose-dependent manner as compared to the control group [Figure 4a and b]. This finding suggests that the modulatory effect of curcumin on core circadian genes might be through the regulation of their associated miRNAs.

DISCUSSION

In most of the neurodegenerative diseases, disruption in the circadian rhythms is noted as a primary symptom. The underlying molecular mechanisms of the circadian system are purported to play an important causal role in the development of neurodegenerative diseases.^[8] Several pilot studies have suggested that disruption in circadian rhythm is not only a symptom of neurodegenerative disease, but it also can be a hypothetical risk factor contributing to its development.^[23] Even though many researchers have suggested the underlying molecular pathways that involve changes in protein homeostasis, immune and provocative functions, the underlying molecular mechanism that connects the circadian rhythms and behavior impairment is still not completely known.

Our present study revealed that the treatment of different concentrations of curcumin for 3 weeks in Swiss albino mice led to a significant improvement in both cognitive (spatial learning, retention memory, and recognition memory) and noncognitive behavior (locomotor activity and anxiety-like behavior). Corroborated to our study, many other studies have suggested that treatment of curcumin improves the behavior of mice including locomotor activity, spatial learning and retention memory, recognition memory, stress, and depressive-like behavior.^[13,15]

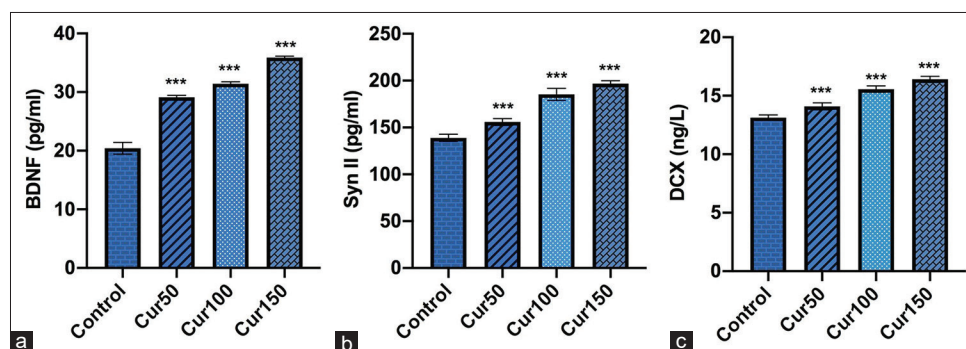


Figure 1: Effect of curcumin on hippocampal proteins level (a) BDNF, (b) Synapsin II, and (c) DCX. Values are represented as mean $\pm$ standard deviation ($n = 7$). One-way ANOVA with a post hoc Duncan's test was employed to compare the results between control versus curcumin-treated groups. The data were analyzed using GraphPad Prism-8. The values of *** $P < 0.001$ represent a statistically significant difference between the groups

In our earlier study, we have also observed that curcumin treatment reduced oxidative stress in cadmium treated mice.^[24] This is observed by other researchers as well.^[25] Since oxidative stress plays a major role in neurodegeneration and many previous studies have reported the oxidative stress-induced behavior impairment in mice,^[26] the current findings and supportive data of past studies suggest that the improvement of behavior in curcumin-treated mice was mediated through reduced oxidative stress by increasing antioxidant enzyme (SOD and CAT) and decreasing the pro-oxidant (MDA).

To strengthen the hypothesis of curcumin as a neuroprotective herbal drug, we also investigated the effect of curcumin on

hippocampal proteins and their mRNA expression level. The findings indicate that the treatment of curcumin for 3 weeks had significantly increased the level of hippocampal proteins and their mRNAs in a dose-dependent manner. The hippocampal proteins including BDNF, Synapsin II, DCX, and CREB play an important role in the regulation of dentate gyrus neurogenesis.^[27] In our previous study, we have also observed curcumin treatment increases the level of these proteins in mice exposed to dim light at night.^[28] Other studies have also reported the same in their experiments.^[29] Therefore, based on our findings and past reports, the improvement of behavior in the curcumin-treated mice was possibly mediated through the promotion of hippocampal neurogenesis.

While the role of disrupted circadian rhythms in neurodegenerative diseases was previously reported,^[30] we investigated the effects of curcumin on core circadian genes (*SIRT1*, *BMAL1*, and *CLOCK*). The results revealed that curcumin indeed significantly modulates the expression of these genes. In the healthy individuals, the expression of these clock genes was in a rhythmic control, modulating many other peripheral genes including the hippocampal genes that we have discussed above. Another study had reported that curcumin has the potential to modulate the expression of circadian genes in the mice model of cancer.^[31] Based on our result and past reports, curcumin could be used as a novel therapeutic drug to manage the behavior impairment in patients with neurodegenerative disorders through modulating the expression of core circadian genes.

The role of miRNAs in brain development is a recent field of study. Many miRNAs regulate the expression of circadian genes in CNS. Therefore, to elucidate the role of miRNAs in CNS clock gene modulation, we investigated the expression level of two circadian clock gene associated miRNAs including *CLOCK* gene-associated miRNA21 and *SIRT1* gene-associated miRNA34. Our results revealed that the treatment of curcumin indeed increased the expression levels of these two miRNAs in a dose-dependent manner. However, there are no previous studies that have particularly reported the effect of curcumin on the expression levels of these miRNAs. This finding might suggest that alteration

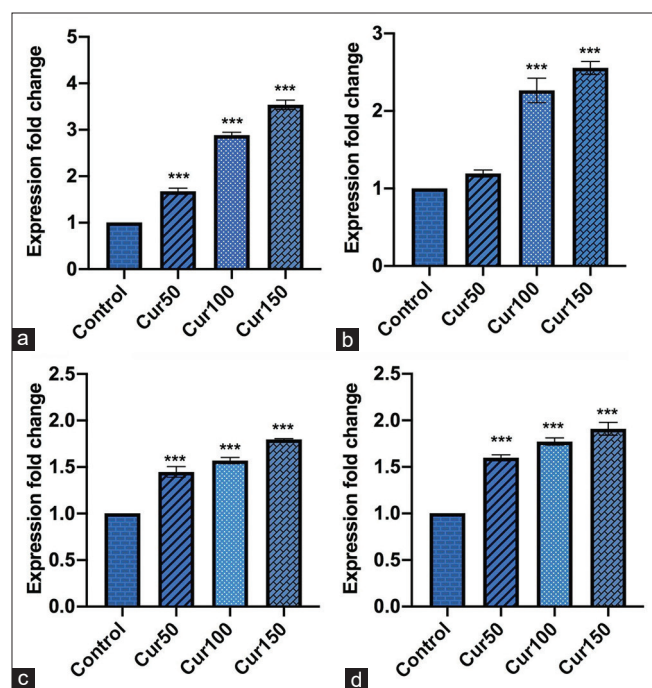


Figure 2: Effect of curcumin on hippocampal mRNA expression level (a) BDNF mRNA, (b) Synapsin II mRNA, and (c) DCX mRNA, and (d) CREB mRNA. Values are represented as mean $\pm$ standard deviation ($n = 7$). One-way ANOVA with a *post hoc* Duncan's test was employed to compare the results between control versus curcumin-treated groups. The data were analyzed using GraphPad Prism-8. The values of *** $P < 0.001$ represent a statistically significant difference between the groups

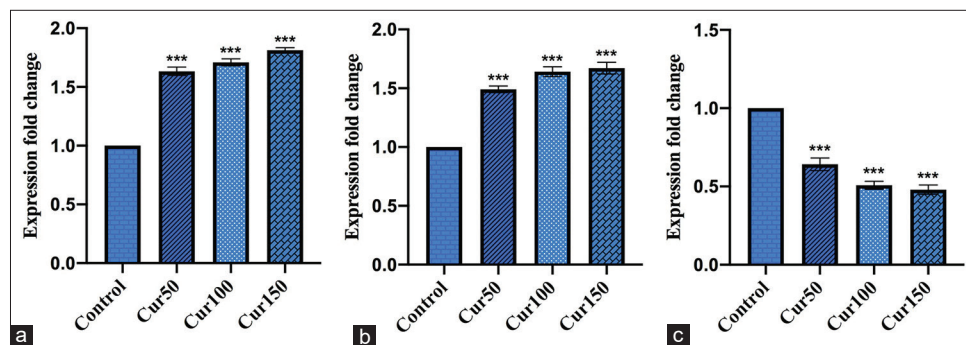


Figure 3: Effect of curcumin on circadian gene (a) *SIRT1*, (b) *BMAL1*, and (c) *CLOCK*. Values are represented as mean $\pm$ standard deviation ($n = 7$). One-way ANOVA with a *post hoc* Duncan's test was employed to compare the results between control versus curcumin-treated groups. All the data were analyzed using GraphPad Prism-8. The values *** $P < 0.001$ represent a statistically significant difference between the groups

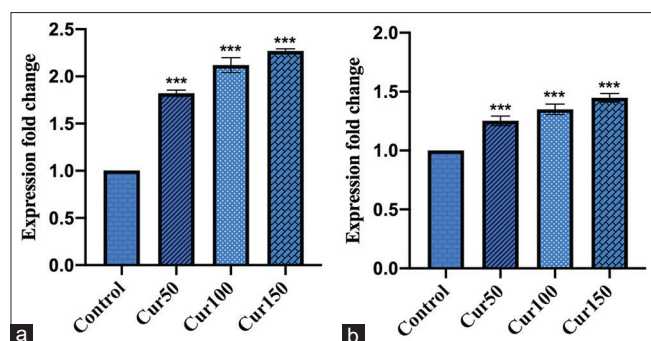


Figure 4: Effect of curcumin on hippocampal miRNA (a) miRNA21a-5p and (b) miRNA34a-5p. Values are represented as mean $\pm$ standard deviation ($n = 7$). One-way ANOVA with a post hoc Duncan's test was employed to compare the results between control versus curcumin-treated groups. The data were analyzed using GraphPad Prism-8. The values *** $P < 0.001$ represent a statistically significant difference between the groups

in the expression of clock genes of the curcumin-treated group could be through modulating the expression of their associated miRNAs.

CONCLUSION

Overall, our current findings suggest that curcumin can be used as a circadian-oriented natural therapeutic drug to improve the behavioral impairment in patients with neurodegenerative disorders through regulation of oxidative stress, hippocampal neurogenesis, and expression level of core circadian genes. Indeed, currently, there are several other circadian-oriented therapeutic approaches toward the treatment of behavior impairment in neurodegenerative disorders, but alongside, if a judicious dose of curcumin could be administered, the recovery and improvement might be more intense and observable.

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Conflicts of interest

There are no conflicts of interest.

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